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SPECIFICATION

TITLE OF INVENTION

An automated method of metabolic stability analysis of library of compounds by isotope dilution mass spectrometry

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CROSS-REFERENCE TO RELATED APPLICATIONS

Not applicable

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable

BACKGROUND OF THE INVENTION (Currently amended)

[0001] This invention pertains to a method of synthesis of labeled internal standards for the quantitative analysis by mass spectrometry (MS) the extent of degradation of libraries of chemical compounds that are treated with liver enzymes in a metabolic stability test. The method includes distinct methodologies to synthesize chemicals required for the MS analysis of these chemical compounds. [~~library of organic compounds by isotope dilution mass spectrometry (MS), particularly in a metabolic stability assay wherein said compounds are treated with enzymes of the liver to determine the extent of metabolism. Said compounds are usually drug candidates resulting from high throughput syntheses~~].

[0002] Metabolic stability test is an *in vitro* test to measure the extent of degradation, or frequently referred as metabolism, of a chemical compound, usually a drug candidate, upon its exposure to enzymes of the liver. An *in vitro* test is a test that is performed outside of an animal or a human being but in an environment that is closest to the biological systems. In practice the metabolic stability test is carried out in aqueous media containing enzymes that are isolated from the livers of recently deceased people or deceased animals. These enzymes are colored (“chrome”) cellular (“cyto”) proteins that possess “pigment at 450nm”, so they are called “Cytochrome P450 enzymes”. The liver is regarded as an important organ responsible for the metabolism of many drug candidates. A poorly degraded or poorly metabolized drug candidate can cause toxic effects and makes it unsuitable or unsafe as a drug. Current practice of metabolic stability testing of chemicals as drug candidates includes reactions of known quantities of these chemicals with known quantities of Cytochrome P450 enzymes for different time intervals. These enzyme reactions are then stopped by addition of an organic solvent to kill the enzyme action, resulting in "stopped enzyme reactions". The "remainder" of these chemicals in the stopped enzyme reactions, or the portions that are not degraded, are determined by MS using internal standards as a mean for quantification. [~~In searching for new drugs for a particular disease, scientists usually start from a lead compound and synthesize several hundreds of analogs for testing in a process called high throughput synthesis. These analogs, or chemical compounds with different chemical structure variations from the lead compound, constitute a so-called "library" of drug candidates.~~]

Start of a delete paragraph: With careful synthesis planning, scientists can come up with a number of good candidates for the preliminary testing of efficacy . The good candidate compounds are then subjected to different assays with animal and human tissues to determine their safety as pharmaceuticals. Metabolic stability assay is an assay used to determine the extent of metabolism of a drug candidate compound when it passes through the liver. Said assay measures the remainder of a candidate compound as a percentage of its original concentration after its contact with the enzymes of the liver. The data from the assay will tell whether a drug candidate is extensively metabolized or poorly metabolized within a certain period of time in the body. **End of a delete paragraph.**

Start of a delete paragraph: Metabolism is the mechanism by which the body uses to eliminate a drug substance. Because all body fluids are aqueous, said method of elimination, or metabolism, is the conversion of drugs into water soluble compounds. Enzymes in the liver are responsible for this conversion. Said enzymes of the liver are usually called the cytochrome P450 enzyme systems. Sources of enzymes include crude liver tissues, microsomes, S9 fractions, and individual isozymes. Said enzyme actions include hydroxylation, demethylation, ester hydrolysis, etc., wherein drugs are converted to the so-called "metabolites" which are drugs that are hydroxylated, demethylated, hydrolyzed, etc. Even though metabolites are no longer drugs, they are still structurally similar to drugs. Therefore, a reliable metabolic stability assay must include an analysis method that can positively identify and accurately quantitate a drug candidate among all of its structurally similar metabolites in the enzyme reaction medium. Mass spectrometric (MS) method is currently a method of choice for said analysis. **End of a delete paragraph.**

Start of a delete paragraph: Said MS method identifies a drug candidate by its molecular weight ion and/or its daughter ion and measures its concentration by quantitation of these ions. To ease quantitation an internal standard is added to crude enzyme reactions after termination of reactions. Quantitation is made based on ratios of ions of drugs and said internal standard. Examples of metabolic stability assays using only one internal standard for the entire library of drug candidates are common. Usually no calibration curve is constructed and no extraction method for crude enzyme reactions is performed. **End of a delete paragraph.**

Start of a delete paragraph: Analysis of crude enzyme reactions most often includes a centrifugation step to separate the macromolecules such as protein from the aqueous part. Without this protein separation step, analysis of the crude enzyme reactions will result in poor signal and build up solvent pressure. Automated centrifugation is difficult, but sample clean up by centrifugation is not all that desirable and. If an extraction step is performed instead of a centrifugation step, the resulting mass spectrometric signal is usually much better. But sample clean up by extraction does have a disadvantage, that is, it is difficult to work out an extraction method wherein good recoveries of the internal standard and all drug candidates in the library are achieved. However, if stable isotope internal standard for each drug candidate is used, then good recoveries will not be necessary . The present invention discloses a metabolic stability assay using MS analysis which includes an automated synthesis of stable isotope labeled internal standards, an automated enzyme reaction protocol, an automated extraction method of crude enzyme reactions, and an MS analysis of said extracts. **End of a delete paragraph.**

Start of a delete paragraph: A mass spectrometric analysis method using stable isotope labeled internal standard(s) is commonly called isotope dilution mass spectrometry. This method takes advantage of the similar chemical and physical behaviors of analytes and their respective isotope labeled internal standards towards all phases of sample preparation (extraction, derivatization, etc.) and also towards instrument responses (similar retention time, similar signal response, etc.). It uses the mass differentiation between analytes and their respective internal standard for the quantitation of the ions. In a nutshell, a metabolic stability assay using isotope dilution MS analysis will provide accurate results regardless of the extraction recoveries of the drug candidate compounds and their labeled internal standards. All it requires is the availability of stable isotope labeled internal standard for each drug candidate compound. **End of a delete paragraph.**

Start of a delete paragraph: The commonly used stable isotope labeled internal standard of an analyte is a chemical compound that has the same chemical structure as that of the analyte except that one or more substituent atoms are stable isotopes (deuterium, C-13, N-15, O-18). Synthesis of individual isotope labeled internal standards for said metabolic stability assay is an enormous task. The automated synthesis would be almost impossible. However, there are alternative ways to achieve the same objective. The objective is a short, reliable, and automated method of synthesis of stable isotope labeled internal standards that is suitable for the analysis of the compound library, but not the synthesis of stable isotope labeled drug candidates. **End of a delete paragraph.**

Start of a delete paragraph: Isotope dilution mass spectrometric analysis requires that both analyte and its internal standard must have identical chemical structures, with the exception of the isotope atoms which provide the mass differentiation for quantitation. Said requirement ensures same extraction recoveries for both and same instrumental responses for both. If both said analyte and its stable isotope labeled internal standard are reacted with the same chemical reagent before analysis, then the products of said reactions must be structurally identical. This type of reaction is common in isotope dilution gas chromatography-mass spectrometry analysis (GC-MS) wherein the reagent used is usually called "derivatizing reagent". The analysis becomes the analysis of the"derivatized" analyte and the "derivatized" internal standard, but their ion ratios remain unchanged. Automated metabolic stability assay using isotope dilution MS analysis method is feasible if both drug candidate and its internal standard are converted to compounds of identical structure, except the labeled atoms, before analysis. If said conversion is performed before extraction from the reaction medium, then their extraction recoveries will be the same. The present invention discloses said conversion in an automated fashion as a method to convert drug candidate compounds to compounds of identical structure as that of said isotope labeled internal standards in reaction medium before extraction wherein said labeled internal standards are already added. **End of a delete paragraph.**

Start of a delete paragraph: Using well known efficient chemical conversion methods, stable isotope labeled internal standards are synthesized in an automated fashion from a portion of the drug candidate compounds and with only one stable isotope labeled chemical reagent. While the enzyme reactions of another portion of said drug candidate compounds are being carried out, said labeled internal standards are isolated by an automated extraction method and then added to appropriate enzyme reactions after they are terminated. A non-labeled version of said chemical reagent is added to said terminated enzyme reactions to transform said candidate compounds into compounds of identical structure, except the labeled atoms, as those of said added labeled internal standards. An automated extraction step for the crude enzyme reactions follows, and the final extracts are then analyzed by mass spectrometry. **End of a delete paragraph.**

Start of a delete paragraph: Said efficient chemical conversion methods are selected based on the type of functional group present in said drug candidate compounds. For example, for said drug candidate compounds that have either a primary or a secondary amino group, said efficient conversion method is the transformation to an acetamide by using an excess of acetic acid anhydride. Said conversion is a one-step simple operation and can be performed in an automated fashion. One chemical reagent such as acetic acid anhydride-d6 can convert all the primary and secondary amino group containing drug candidate compounds in an automated fashion to acetamide-d6 analogs. The excess acetic anhydride-d6 is destroyed and said acetamide-d6 analogs are separated by an automated extraction and then added to enzyme reactions after they are terminated. Said terminated enzyme reactions are then, in an automated fashion, treated with excess acetic acid anhydride to transform said drug candidate compounds to acetamide analogs. Said acetamide-d6 analog in each terminated enzyme reaction is unreactive toward acetic acid anhydride. Both said acetamide-d6 analogs and said acetamide analogs in said terminated enzyme reactions are then extracted in an automated fashion, and the extracts are injected into a mass spectrometer for analysis. **End of a delete paragraph.**

Start of a delete paragraph: This invention discloses an automated synthesis method for said drug candidate compounds that contain any of the four following functionality groups :

1. Group 1: a primary or a secondary amino functional group of a primary or a secondary amine compound.
2. Group 2 : an hydroxyl functional group of an alcohol or a phenolic compound.
3. Group 3 : a carbonyl functional group of an aldehyde or a ketone compound.
4. Group 4 : a carboxyl functional group of a carboxylic acid compound.

Said efficient chemical conversion methods include :

For group 1, a primary or a secondary amino functional group :

1. conversion to an amide by using either an acid anhydride or an acid chloride.
2. conversion to a carbamate by using a chloroformate.
3. conversion to an urea by using an isocyanate.
4. conversion to a thiourea by using a thioisocyanate.

For group 2, a phenol or a hydroxyl functional group :

1. conversion to an ester by using either an acid anhydride or an acid chloride.
2. conversion to a carbamate by using an isocyanate.

For group 3, a carbonyl functional group of an aldehyde or a ketone :

1. conversion to an oxime by using an alkoxylamine.
2. conversion to a hydrazone by using an alkylhydrazine.

For group 4, a carboxyl functional group of a carboxylic acid : conversion to a carboxylic ester by using either a combination of alkylchloroformate and alcohol or a combination of base and alkyl halide. **End of a delete paragraph.**

Start of a delete paragraph: Said efficient conversion reactions are selected based on the following criteria

1. Said candidate compounds in said terminated enzyme reactions must be ***quantitatively*** converted to the compound of identical structure, except the labeled atoms, as that of said added isotope labeled internal standards using a non-labeled reagent.
2. Absolutely no conversion of said isotope labeled internal standard to said non-labeled analog because the conversion of said candidate compound occurs in said reaction medium in the presence of said added isotope labeled internal standard.
3. The conversion of said candidate compound into said compound of identical structure as that of said added isotope labeled internal standard has to be accomplished ***before*** extraction, not after extraction as in many cases of derivatization of GC-MS methods.

There are other conversion reactions that are very efficient, but said conversion reactions are very efficient in aqueous environment and can be performed at room temperature and in a relatively short reaction time. Said features are necessary for the invented automated metabolic stability assay using isotope dilution MS analysis. **End of a delete paragraph.**

[0003] **Added paragraph:** It has been shown in chemical literature and particularly in cited references that MS analysis of compounds in biological samples provides reliably accurate result with the use of stable isotope labeled internal standards. "Stable isotope labeled internal standard" used for MS analysis of a chemical compound is a compound that has the exact chemical structure as that of the chemical compound except that some of its constituent atoms including hydrogen, carbon, nitrogen, and oxygen are enriched with the stable isotope atom deuterium, carbon-13, nitrogen-15, and oxygen-18, respectively. Because no radioactive or unstable isotope atom is used in MS analysis, so "labeled internal standard" is usually referred to stable isotope labeled internal standard. Due to nature of stable isotope, labeled internal standard behaves the same chemical and physically as the chemical compound being analyzed towards sample processing and analysis. The mass spectrometer instrument, however, can recognize the chemical compound and its labeled internal standard as two different ion signals because they have different masses resulting from the difference in the number of enriched isotope atoms. So accurate determination of concentration of the chemical compound can be made by calculation using known concentration of the labeled internal standard and the ion signals of both compounds. Since samples of enzyme reactions in a metabolic stability test are also biological samples. Therefore an accurate MS determination of concentration of the remainder of drug candidate compounds in enzyme reactions preferably requires the use of labeled internal standards.

[0004] **Added paragraph:** Libraries of drug candidate compounds are classes of chemical compounds that have a common reactivity towards certain type of receptors in the body. Libraries of drug candidate compounds are created by chemical synthesis, most often from a "lead" compound which is a chemical compound having some positive activities against a disease. Using the structure of this lead chemical compound, chemists synthesize other chemical compounds having structures deviated from the structure of the lead compound but still retaining major functional features that are crucial for reactivity. All the synthesized chemical compounds constitute a library of chemical compounds or library of drug candidates. This common reactivity is usually due to the presence of at least one functional group in common in each member including amino groups (-NH_2 or –NH-), hydroxyl groups (-OH), carbonyl groups (-CO-), and carboxyl groups (-COOH). These common functional groups in all members allow them to engage into special chemical bonding characteristic of each library. Reactivity towards certain receptor is regarded as one type of chemical bonding which provides attachment of the chemical to the "defective"receptor to restore its normal function, thereby curing the disease. Metabolism is regarded as another type of chemical bonding that these library compounds engage in, turning them into metabolites which are products of these bonding reactions. Metabolic stability test measures to what extent the library compounds transformed into metabolites. The test does not measure the metabolites but it measures how much of the library compounds remained after their exposure to Cytochrome P450 enzymes.

[0005] **Added paragraph:** It has been shown in the chemical literature that a chemical method called "derivatization" can provide better signal response in MS analysis of chemical compounds in samples of biological origins. This derivatization method involves treatment of a chemical compound whose concentration is to be determined with a "derivatizing reagent" to " tag on" or "chemically attach" a chemical group to the existing functional group of the chemical compound. The resulting "derivatized chemical compound" now produces better MS signal response due to the presence of this "tagged chemical group". There are derivatizing reagents that react with only one functional group and there are also derivatizing reagents that react with more than one functional group. The reaction of these derivatizing reagents with appropriate functional groups are often very efficient that they can be applied to a variety of structurally different chemical compounds without much reaction condition optimization required, even in aqueous biological samples. Many derivatization reactions are "100% quantitative", meaning all of the reactants are completely converted to derivatized product under a given set of reaction conditions. An example of the use of the derivatizing reagent ethyl chloroformate in combination with ethanol and pyridine base in MS analysis of homocysteine is demonstrated by Jens Pietzsch et al. One chemical group -C_2H_5 is tagged on to the carboxyl function –COOH and one chemical group –$CO_2C_2H_5$ is tagged on to both the amino function –NH_2 and the thiol function –SH of homocysteine. This MS analysis of homocysteine did provide a high sensitivity analysis as a result of derivatization. The use of the derivatizing reagent ethyl chloroformate-ethanol combination in this case produces an MS ion signal that is higher in mass in comparison to that of homocysteine. This mass difference is equivalent to one -C_2H_5 and two –$CO_2C_2H_5$, the mass of three tagged chemical groups. This particular example and other examples in cited references shows that the choice of derivatizatizing reagents is usually not limited to the type of functional groups present in a chemical compound. The type of functional group serves as a guide for the selection of derivatizing reagents which in turn provide information about the type of anticipated tagged chemical group. MS instruments do not differentitate chemical compounds based on their functional groups but rather on the mass of the chemical compounds. Derivatization always results in an increase in mass of a chemical compound, and this is due to the tagged chemical group. Using the derivatization method, the MS analysis of a chemical compound becomes the analysis of the "derivatized chemical compound", which is the reaction product of the chemical compound and the derivatizing reagent. When a labeled derivative of a chemical compound for use as internal standard is added to sample of the chemical compound being analyzed, both the chemical compound and its labeled internal standard are derivatized upon reaction with a derivatizing reagent resulting in the chemical attachment of the same tagged chemical group to both compounds. Concentration of the derivatized chemical compound in all cases is regarded as the concentration of the chemical compound being analyzed when both the derivatized chemical compound and its derivatized labeled internal standard are present in the final extract.

[0006] **Added paragraph:** With the increasing number of syntheses of libraries of drug candidate compounds, the demand for high throughput metabolic stability analysis is high. New instruments for automation are manufactured, there is a demand for accurate method of metabolic stability analysis that can be adapted to automated instruments. Metabolic stability test can be automated but accurate result entails an availability of labeled internal standard of each library member for MS analysis, a difficult scenario for most metabolic stability test. Synthesis of all members of a library of chemical compounds from a lead compound is a tremendous effort. The synthesis of labeled derivative of each member for use as internal standard is even a bigger task. There is a need to develop short synthesis method for labeled chemical compound that can be used as labeled internal standard for each library member, preferably in a simultaneous fashion so that this method can be incorporated into a metabolic stability test adapted to automated instruments.

BRIEF SUMMARY OF THE INVENTION (Currently amended)

[0007] As increasing number of libraries of new drug candidates become available, methods to accurately assess their safety for clinical trials are becoming more demanding especially methods that can be adapted to automated instruments. The objective of the invention is to provide a method of one step simultaneous synthesis of labeled chemical compounds that may be used as labeled internal standards in the MS determination of concentration of all members of a library of chemical compounds after their reaction with Cytochrome P450 enzymes. The objective of the invention is also to provide additional chemical reaction that is required when using these labeled compounds as labeled internal standards. The objective of the invention is also to develop this additional reaction into one step simultaneous reaction that can be incorporated into reactions of all library members with cytochrome P450 enzymes, preferably adapted to automated instruments. The following detailed description of the invention, the mass spectra of labeled and non labeled derivatized chemical compounds of the example, and the claims will further clarify the objective of the invention. [~~The subject of the current invention provides for an automated method of metabolic stability analysis of a library of compounds. Said automated analysis include the following sequences :~~

~~1. automated synthesis and extraction of stable isotope labeled internal standards,~~

~~2. automated synthesis and extraction of non-label version of said internal standards for establishing parameters for mass spectrometric analysis,~~

~~3. automated reactions of cytochrome P450 enzyme systems with library compounds,~~

~~4. automated termination of said enzyme reactions and addition of said internal standards to said terminated reactions,~~

~~5. automated conversion of said library compounds in said enzyme reactions to compounds of identical structure as that of said internal standards,~~

~~6. automated extraction of said terminated enzyme reactions,~~

~~7.~~ ~~mass spectrometric analysis of said internal standards, said non-labeled version of said internal standards, and said enzyme reactions, and~~

8. ~~calculation of percentage of remainder of library of compounds after enzyme reactions.~~]

BRIEF DESCRIPTION OF THE DRAWINGS (New)

[0008] Figure 1 illustrates a mass spectrum of the synthesized labeled internal standards, acetamides-d3, of the library of 4 amines in molecular ion mode according to the example of the detailed description of the invention.

Figure 2 illustrates a mass spectrum of the derivatizized library compounds, acetamides, of the library of 4 amines in molecular ion mode according to the example of the detailed description of the invention.

Figure 3 illustrates a mass spectrum of the synthesized labeled internal standards, acetamides-d3, of the library of 4 amines in MRM mode according to the example of the detailed description of the invention.

Figure 4 illustrates a mass spectrum of the derivatizized library compounds, acetamides, of the library of 4 amines in MRM mode according to the example of the detailed description of the invention.

Figure 5 illustrates a mass spectrum of the extract of a stopped enzyme reaction containing the derivatizized library compounds, acetamides, and the synthesized labeled internal standards, acetamides-d3, of the library of 4 amines in MRM mode according to the example of the detailed description of the invention.

DETAILED DESCRIPTION OF THE INVENTION (Currently amended)

[0009] [~~The method of the subject invention provides an automated metabolic stability assay and a quantitative analysis of library compounds after reactions with cytochrome P450 enzyme systems using isotope dilution mass spectrometric analysis. Chronologically, depending on the type of functional group present in said library compounds, the invention provides~~

~~methods of synthesis of stable isotope labeled internal standards of said library compounds using only one type of labeled chemical reagent,~~

~~methods of extraction of said internal standards,~~

~~methods of synthesis of a non-labeled version of said internal standards using the non-labeled version of said labeled chemical reagent,~~

~~methods of extraction of said non-labeled version of said internal standards for use in setting up parameters for mass spectrometric analysis,~~

~~methods of reactions of enzymes of the cytochrome P450 enzyme systems with library compounds,~~

~~methods of termination of enzyme reactions and addition of said extracts of said stable isotope labeled internal standards to said terminated enzyme reactions,~~

~~methods of conversion of remaining library compounds in said terminated enzyme reactions to compounds of identical structure as that of said internal standards using said non-labeled version of said labeled chemical reagent,~~

~~methods of extraction said internal standards and said converted compounds from said reaction medium,~~

~~methods of determination of molecular ion and product ion (also called daughter ion) of said internal standards and said converted compounds in said chemical synthesis extracts,~~

~~methods of selection of ion ratios of said molecular ions and said daughter ions and use of said ion ratios in MRM mode (multiple reaction monitoring mode),~~

~~methods of mass spectrometric analysis of said extracts of enzyme reactions in MRM mode,~~

~~methods of using mass spectrometric data of said selected ion ratios to determine percent remaining of said library compounds from said enzyme reactions.~~]The current invention provides a method of one step simultaneous synthesis of labeled compounds that can be used as labeled internal standards in the MS analysis of the remainder of a library of chemical compounds in a metabolic stability test that can be adapted to automated instruments. These labeled compounds are synthesized by a single step reaction of a labeled chemical reagent having at least one enriched stable isotope atom, called "labeled derivatizing reagent", with an authentic sample of all members of that library of chemical compounds. Therefore, the reaction is called a "one step simultaneous synthesis". The products of this reaction all have the same labeled tagged chemical group coming from the one step reaction of this labeled derivatizing reagent. The method of this invention is directed to the use of these labeled compounds as labeled internal standards in the MS analysis of library compounds after their exposure to Cytochrome P450 enzymes. This method is possible only in cases where library compounds have in common at least one of the four functional groups including amino groups represented as R_2NH , hydroxyl groups represented as ROH, carbonyl groups represented as R_2CO, carboxyl groups represented as RCOOH, wherein R is part of the library compounds composed of hydrogen, alkyl, aryl, hetero atom containing, cyclic, non-cyclic groups. Even though the type of common functional group present in library compounds dictates the possible one step simultaneous reaction with the labeled derivatizing reagent, it is the selection of labeled derivatizing reagent that dictates the type of labeled tagged chemical group to be chemically attached to the common functional group.

Start of a delete paragraph: Specifically, the method of the subject invention provides methods of synthesizing stable isotope labeled amide, carbamate, urea, and thiourea internal standards of primary and secondary amine compounds by reacting said compounds with a stable isotope labeled chemical reagents selected from a group consisting of labeled acid anhydrides or labeled acid chlorides, labeled chloroformates, labeled isocyanates, and labeled thioisocyanates. The invention provides methods of converting said amine compounds in said terminated enzyme reactions into compounds of identical structure as that of said labeled internal standards by adding to said terminated reactions a non-labeled version of said chemical reagents selected from a group consisting of non-labeled acid anhydrides or non-labeled acid chlorides, non-labeled chloroformates, non-labeled isocyanates, and non-labeled thioisocyanates. **End of a delete paragraph.**

Start of a delete paragraph: Specifically, the method of the subject invention provides methods of synthesizing stable isotope labeled ester and carbamate internal standards of phenolic or alcoholic compounds by reacting said compounds with a stable isotope labeled chemical reagents selected from a group consisting of labeled acid anhydrides or labeled acid chlorides, and labeled isocyanates. The invention provides methods of converting said phenolic or alcoholic compounds in said terminated enzyme reactions into compounds of identical structure as that of said labeled internal standards by adding to said terminated reactions a non-labeled version of said chemical reagents selected from a group consisting of non-labeled acid anhydrides or non-labeled acid chlorides, and non-labeled isocyanates. **End of a delete paragraph.**

[0010] **Added paragraph :** Specifically the objective of the present invention provides methods of one step simultaneous reaction of an authentic sample of library compounds with labeled derivatizing reagents acid anhydrides $(R^*CO)_2O$ or acid chlorides R^*COCl to attach a labeled tagged chemical group R^*CO to existing primary amino group and secondary amino group R_2NH, and hydroxyl group ROH of library compounds resulting in derivatized chemical compounds R^*CONR_2 and R^*COOR, respectively. Specifically the objective of the present invention provides methods of one step simultaneous reaction of an authentic sample of library compounds with labeled derivatizing reagent alkyl chloroformates R^*OCOCl to attach a labeled tagged chemical group R^*OCO to existing primary amino group and secondary amino group R_2NH, and hydroxyl group ROH of library compounds resulting in derivatized chemical compounds R^*OCONR_2 and R^*OCOOR, respectively. Specifically the objective of the present invention provides methods of one step simultaneous reaction of an authentic sample of library compounds with labeled derivatizing reagent isocyanates R^*NCO to attach a labeled tagged chemical group R^*NHCO to existing primary amino group and secondary amino group R_2NH, and hydroxyl group ROH of library compounds resulting in derivatized chemical compounds $R^*NHCONR_2$ and $R^*NHCOOR$, respectively. Specifically the objective of the present invention provides methods of one step simultaneous reaction of an authentic sample of library compounds with labeled derivatizing reagent thioisocyanates R*NHCS to attach a labeled tagged chemical group R*NHCS to existing primary amino group and secondary amino group R_2NH, and hydroxyl group ROH of library compounds resulting in derivatized chemical compounds $R^*NHCSNR_2$ and R*NHCSOR, respectively. R* is a labeled alkyl group such as CD_3 and $^{13}CD_3$ or a labeled aryl group such as C_6D_5 and $^{13}C_6H_5$. The derivatized library compounds having the molecular mass increased by the above labeled tagged chemical group R*CO , R*OCO , R*NHCO , R*NHCS are suitable for use as labeled internal standards in the MS analysis of the remainder of the library compounds in stopped enzyme reactions.

[0011] [~~Specifically, the method of the subject invention provides methods of synthesizing stable isotope labeled oxime and hydrazone internal standards of aldehyde or ketone compounds by reacting said compounds with a stable isotope labeled chemical reagents selected from a group consisting of labeled alkoxylamines, and labeled alkylhydrazines. The invention provides methods of converting said aldehyde and ketone compounds in said terminated enzyme reactions into compounds of identical structure as that of said labeled internal standards by adding to said terminated reactions a non-labeled version of said chemical reagents selected from a group consisting of non-labeled alkoxylamines and non-labeled alkylhydrazines.~~] Specifically the objective of the present invention provides methods of one step simultaneous reaction of an authentic sample of library compounds with labeled derivatizing reagent alkoxylamine R^*ONH_2 to attach a labeled tagged chemical group R*ON to existing carbonyl group R_2CO of library compounds resulting in derivatized chemical compounds R^*ONCR_2. Specifically the objective of the present invention provides methods of one step simultaneous reaction of an authentic sample of library compounds with labeled derivatizing reagent alkylhydrazine R^*NHNH_2 to attach a labeled tagged chemical group R*NHN to existing carbonyl group R_2CO of library compounds resulting in derivatized chemical compounds R^*NHNCR_2. R* is a labeled alkyl group such as CD_3 and $^{13}CD_3$ or a labeled aryl group such as C_6D_5 and $^{13}C_6H_5$. The derivatized library compounds having the molecular mass increased by the above labeled tagged chemical group R*ON and R*NHN are suitable for use as labeled internal standards in the MS analysis of the remainder of the library compounds in stopped enzyme reactions.

[0012] [~~Specifically, the method of the subject invention provides methods of synthesizing stable isotope labeled ester internal standards of carboxylic acid compounds by reacting said compounds with either a stable isotope labeled alcohol or a labeled alkyl halide. The invention provides methods of converting said carboxylic acid compounds in said terminated enzyme reactions into compounds of identical structure as that of said labeled internal standards by adding to said terminated reactions a non-labeled version of said labeled alcohol or said labeled alkyl halide.~~] Specifically the objective of the present invention provides methods of one step simultaneous reaction of an authentic sample of library compounds with labeled derivatizing reagent alcohol R*OH and a catalyst alkylchloroformate to attach a labeled tagged chemical group R* to existing carboxyl function RCOOH of library compounds resulting in derivatized chemical compounds R*OOCR. Specifically the objective of the present invention provides methods of one step simultaneous reaction of an authentic sample of library compounds with labeled derivatizing reagents alkyl chloride R*Cl, alkyl bromide R*Br, alkyl iodide R*I and a catalyst base such as sodium hydroxide to attach a labeled tagged chemical group R* to existing carboxyl function RCOOH of library compounds resulting in derivatized chemical compounds R*OOCR. R* is a labeled alkyl group such as CD_3, $^{13}CD_3$, $C_6D_5CH_2$, and $^{13}C_6H_5\,CH_2$. The derivatized library compounds having the molecular mass increased by the above labeled tagged chemical group R* are suitable for use as labeled internal standards in the MS analysis of the remainder of the library compounds in stopped enzyme reactions. The following table summarizes the labeled derivatizing reagents, the functional groups, the anticipated labeled tagged chemical groups, and the derivatized chemical compounds.

Labeled deri. reagent	***Funct. group***	***Tagged group***	***Der. cpds***
$(R*CO)_2O$	R_2NH, ROH	R*CO	$R*CONR_2$, R*COOR
R*COCl	R_2NH, ROH	R*CO	$R*CONR_2$, R*COOR
R*OCOCl	R_2NH, ROH	R*OCO	$R*OCONR_2$,R*OCOOR
R*NCO	R_2NH, ROH	R*NHCO	$R*NHCONR_2$,R*NHCOOR
R*NCS	R_2NH, ROH	R*NHCS	$R*NHCSNR_2$,R*NHCSOR
$R*ONH_2$	R_2CO	R*ON	$R*ONCR_2$
$R*NHNH_2$	R_2CO	R*NHN	$R*NHNCR_2$
R*OH	RCOOH	R*	R*COOR
R*Cl	RCOOH	R*	R*COOR
R*Br	RCOOH	R*	R*COOR
R*I	RCOOH	R*	R*COOR

R* is CD_3, $^{13}CD_3$, C_6D_5, $^{13}C_6H_5$, $C_6D_5CH_2$, and $^{13}C_6H_5CH_2$

[0013] **Added paragraph:** The objective of the current invention is also to provide methods of isolation of labeled internals standards by aqueous extraction so that the labeled internal standards are free of excess labeled derivatizing reagents. Aqueous extraction method may be carried out in one of the 3 formats :

1) liquid liquid extraction wherein organic solvent is used to isolate derivatized compounds from the aqueous solution of enzyme, salts, etc.
2) solid phase extraction wherein solution of derivatized compounds, enzymes, salts, etc is passed through an absorbent material that has the ability to retain preferentially the derivatitized compounds. The retained derivatitized compounds are then extruded by passing an organic solvent through the absorbent.
3) Solid liquid extraction wherein a mixture of organic solvent, aqueous solution of enzyme, derivatized compounds, salts, etc. is passed through an absorbent material that has the ability to retain preferentially the aqueous solution, enzyme, salts, etc, but not the organic solvent and the derivatized compounds.

The method of aqueous extraction to isolate the derivatized labeled internal standards may be programmed after the synthesis, and a volume of the solvent extract may also be programmed to be added to each of the cytochrome P450 enzyme reactions to stop the enzyme activity.

[0014] [~~Specifically, said cytochrome P450 enzyme systems in said metabolic stability assay can be cryopreserved or fresh human and animal hepatocytes, microsomes, S9 fractions or solutions containing individual cytochrome P450 isoenzymes.~~] Performing in vitro enzyme reactions involves programmed addition of solutions of library compounds to reactors containing specific concentration of Cytochrome P450 enzymes at body temperature (37 degree Celcius). These enzymes are previously isolated from livers of recently deceased people or animals and are usually made commercially available for testing. The enzyme systems made available for metabolic stability test can be cryopreserved or fresh human and animal hepatocytes, microsomes, S9 fractions or solutions containing individual Cytochrome P450 isozymes. Isozymes are classes of Cytochrome P450 enzymes that can independently metabolize substrates in a number of different pathways including demethylation, hydroxylation, sulfonation, etc. So measurement of the extent of metabolism in a metabolic stability test can be general or specific depending on the sources of enzyme.

[0015] [~~Specifically, said enzyme reactions include reaction at time zero wherein reaction is terminated immediately after enzyme addition. Said MS data of said time zero reaction are used as original concentration of library compounds.~~] The extent of metabolism of drug candidates in a metabolic stability test is a measure of enzyme reactions at time intervals. A programmed metabolic stability test must include reaction at time zero wherein the reaction with enzymes is stopped immediately as soon as the library compounds are added to solution of enzymes. Other time intervals should be chosen so that reasonable metabolism can be assessed for the whole library of compounds in one experiment. "Stopping" an enzyme reaction is to stop the enzyme activity and this is accomplished by addition of an organic solvent to the enzyme reaction resulting in "stopped enzyme reaction". Action of enzymes can only be sustained in aqueous solution of required salts and buffer. An addition of an organic solvent such as methanol or acetonitrile or ethyl acetate to a concentration of more than 5% the reaction solution is sufficient to disrupt the enzyme action and destroy the enzymes themselves. Hence there is no enzyme activity in "stopped enzyme reactions". The program can be made simpler by stopping the enzyme reactions with a solution containing the organic extract of labeled internal standards.

Start of a delete paragraph: Specifically, said enzyme reactions include reactions at different time intervals wherein each reaction is terminated at specific time after enzyme addition. Said MS data of said time reactions are used to calculate the percentage of remaining of library compounds at said time intervals. **End of a delete paragraph.**

Start of a delete paragraph: Specifically, the method of the subject invention provides methods of extraction of reactions by solid liquid extraction, liquid liquid extraction, and solid phase extraction. **End of a delete paragraph.**

[0016] **Added paragraph:** Accurate MS analysis of the remainder of library compounds after their reaction with Cytochrome P450 enzymes is the objective of this invention. This is accomplished by the use of labeled internal standards which are synthesized by a one step simultaneous reaction of library compounds with a labeled derivatizing reagent. The use of these labeled internal standards requires an additional chemical reaction to be performed with the remainder of the library compounds to convert them to chemical compounds having the exact chemical structure as that of the respective member of those labeled internal standards with the exception of labeled isotope atoms. The additional chemical reaction is the one step simultaneous reaction of the remainder of library compounds with a non labeled version of the labeled derivatizing reagent used for the synthesis of the labeled internal standards, resulting in "derivatized library compounds"having the same but non labeled tagged chemical group as those of the labeled internal standards. The following table summarizes the non labeled derivatizing reagents, the common functional groups, the anticipated tagged chemical groups, and the derivatized library compounds :

Derivatizing reagent	*Funct. group*	*Tagged group*	*Der. cpds*
$(RCO)_2O$	R_2NH , ROH	RCO	$RCONR_2$, RCOOR
RCOCl	R_2NH , ROH	RCO	$RCONR_2$, RCOOR
ROCOCl	R_2NH , ROH	ROCO	$ROCONR_2$,ROCOOR
RNCO	R_2NH , ROH	RNHCO	$RNHCONR_2$,RNHCOOR
RNCS	R_2NH , ROH	RNHCS	$RNHCSNR_2$,RNHCSOR
$RONH_2$	R_2CO	RON	$RONCR_2$
$RNHNH_2$	R_2CO	RNHN	$RNHNCR_2$
ROH	RCOOH	R	RCOOR
RCl	RCOOH	R	RCOOR
RBr	RCOOH	R	RCOOR
RI	RCOOH	R	RCOOR

R is CH_3 , C_6H_5 , and $C_6H_5CH_2$

[0017] **Added paragraph:** The objective of the method is to accurately provide concentration of the remainder of library compounds after enzyme reactions by MS analysis of derivatized library compounds and labeled internal standards. Because the remainder of the library compounds are derivatized in the presence of the added labeled internal standards which are already derivatized, it is essential that the reaction of the remainder of library compounds with the derivatizing reagent be optimized by using excess derivatizing reagent so that the remainder of library compounds are completely converted to derivatized library compounds. This reaction condition is referred as “100% quantitative” meaning all library compounds are completely derivatized. The derivatization reaction has to be an irreversible reaction which means the derivatized chemical compound will not revert back to the reactant under the reaction condition. This is essential because there must be no conversion of the labeled internal standards to the corresponding non labeled compounds or the derivatized library compounds in this case. Both the derivatized library compounds and the labeled internal standards are isolated from the aqueous sample by an aqueous extraction similar to the isolation of the labeled internal standards before MS analysis of both in the final extract. It is the objective of the invention to provide methods of one step additional reaction of the remainder of library compounds that can be adapted to automated instruments.

[0018] Example :

The following example demonstrates the one step synthesis of stable isotope labeled internal standards of a library of 4 chemical compounds having in common an amine functional group R_2NH wherein R_2 are represented as : amine 1, $C_6H_5CH_2CH(CH_3)NH_2$; amine 2, $C_6H_5CH_2CH(CH_3)NH(CH_3)$; amine 3, $(OCH_2O)C_6H_3CH_2CH(CH_3)NH(CH_3)$; amine 4, $(OCH_2O)C_6H_3CH_2CH(CH_3)NH(CH_2CH_4)$. The labeled derivatizing reagent acetic anhrydride-d6 $(CD_3CO)_2O$ is used to derivatize all 4 chemical compounds to labeled compounds with the mass spectrum shown. Each labeled compound is chemically attached with a CD_3CO tagged chemical group resulting in an increase in mass of 45 amu (atomic mass unit). The library compounds are treated with Cytochrome P450 enzymes in a metabolic stability test and the concentration of the remainder of the library compounds in stopped enzyme reactions are determined by MS using the synthesized labeled compounds as labeled internal standards. An additional reaction is required for this MS analysis to convert the remainder of the library compounds in stopped enzyme reaction to compound having the tagged chemical group CH_3CO resulting in an increase in mass of 42amu . This additional reaction is the one step derivatization of the library compounds in stopped enzyme reactions with acetic anhydride $(CH_3CO)_2O$ to compounds having the exact chemical structure as that of the labeled internal standards except the labeled atoms as shown in the mass spectrum of the derivatized library compounds. The MS analysis of the aqueous extract containing both the derivatized library compounds and the labeled internal standards provides the concentration of the remainder of library compounds after Cytochrome P450 enzyme reactions so that an evaluation of the extent of their metabolism can be made. The example demonstrates a process of metabolic stability test that can be adapted to automated instruments including performing enzyme reactions, one step synthesis of labeled internal standards, and one step derivatization of library compounds.

Method : A library of 4 amines was analyzed for their metabolic stability using human microsomal proteins as the cytochrome P450 enzyme system. Aliquots of each amine were automatically placed in test tubes which were used for synthesis of its internal standard, synthesis of reference standards, and reactions with cytochrome P450 enzyme system. The internal standard of each amine is the acetamide-d3 compound formed by reaction of amines with acetic anhydride-d6. At the same time an aliquot of each amine was treated with acetic anhydride to form acetamide reference standard which is used to establish MS parameters for analysis of amines in enzyme reactions. Deuterated acetamide internal standards and acetamides were then separated by an automated extraction procedure. The reactions of amines with human microsomal protein were carried out in test tubes in 37^{0}C bath and was terminated by aspirating equal aliquots of the reaction solutions into test tubes containing equal volume of acetonitrile as terminating reagent. Reactions were aspirated at 0, 30, 60, 90, and 120 minutes. After all reactions were stopped, equal volumes of extracted acetamides-d3 were added to respective reactions as internal standards. Aqueous sodium bicarbonate and acetic anhydride were then added to convert remained amine in each reaction to the acetamide while leaving its acetamide-d3 internal standard unchanged. The tubes were mixed gently for 10 minutes. Both acetate amide and its acetate amide-d3 were separated from the reaction by an automatic extraction procedure. Solutions of extracts containing acetamide-d3, acetamide, and both from reactions with cytochrome P450 enzyme sytem were analyzed by electrospray mass spectrometry. Electrospray mass spectrometry method is a method of mass spectrometry analysis wherein a chemical compound being analyzed is identified by the ion of its molecular mass called molecular ion and also by the ion of the product of the molecular ion with a collision gas called daughter ion. The mode of mass spectrometry analysis wherein only the molecular ion is identified is called molecular ion mode. The mode of mass spectrometry analysis wherein both the molecular ion and the daughter ion are identified is called multiple reaction monitoring mode (MRM mode). Molecular ion and its product ion (daughter ion) of all acetamides and acetamides-d3 were determined in an automated fashion using an autosampler, a mass spectrometer and its analysis software. Mass spectra of molecular ion and daughter ion of acetamide-d3 and acetamide of all 4 amines are shown in Figures 1 to 5. The labeled tagged chemical group CD_3CO increases the mass of the amine by 45 amu while the tagged chemical group CH_3CO increases the mass of the amine by 42 amu. After all interested ions were determined, analysis of reaction extracts in MRM mode followed. The ion ratios of acetate amide to acetate amide-d3 were calculated and plotted against time.

[0019] Experiment :

Details of automated sequences are as follows:

1. **Division of the individual amine into multiple equal amounts**

Via a standard liquid handler, aliquots of 4 amines (amine 1, 2, 3, and 4) were placed in 3 rows of 4 test tubes at volume of 0.005ml and concentration of 1mg/ml in methanol. Row 1 was for synthesis of internal standards, row 2 for synthesis of reference standards, and row 3 for reactions with human microsomal protein.

2. **Synthesis of acetamide-d3 internal standards and acetamide reference standards**

Row 1 test tubes were treated with 0.1ml of 10% v/v acetic anhydride-d6 in ethyl acetate while row 2 test tubes were treated with 0.1ml of 10% v/v acetic anhydride in ethyl acetate. Next, aliquots of 0.1ml 1M sodium bicarbonate were added to both rows 1 and 2. Tubes were mixed and allowed to stand for 10 minutes. Then, reactions were aspirated into filters containing hydromatrix® powder and aliquots of 0.900ml ethyl acetate were added to filters. Filtrates contaning acetate amide-d3 were collected in new row 4 test tubes while filtrates contaning acetate amide were collected in new row 5 test tubes. Each of these filtrate solutions were assumed to be at the concentration of 0.005mg/ml in ethyl acetate.

3. **Reactions of amines with human microsomal protein**

Row 3 test tubes containing 0.005ml of amine at 1mg/ml in methanol were treated with 0.345 ml of 0.1M phosphate buffer pH 7 and 0.025ml of 20mg/ml human microsomal protein. The test tubes were incubated at 37^0C and were added 0.125 ml of buffer containing NADP (1.7mg/ml), glucose-6-phosphate (7.8mg/ml), glucose-6-phosphate dehydrogenase (1.5 units/ml), and sodium bicarbonate (20mg/ml). Immediately at time 0, 0.100 ml of row 3 reactions were aspirated to row 6 test tubes containing 0.100ml acetonitrile each. At time 30 minutes, 0.100 ml of row 3 reactions were aspirated to row 7 test tubes containing 0.100ml acetonitrile each. At time 60 minutes, 0.100 ml of row 3 reactions were aspirated to row 8 test tubes containing 0.100ml acetonitrile each. At time 90 minutes, 0.100 ml of row 3 reactions were aspirated to row 9 test tubes containing 0.100ml acetonitrile each. At time 1200 minutes, 0.100 ml of row 3 reactions were aspirated to row 10 test tubes containing 0.100ml acetonitrile each.

4. **Sample processing of microsome reactions.**

Rows 6,7,8,9, and 10 test tubes contained microsome reactions that was terminated by 0.100ml acetonitrile. An aliquot of 0.050ml solution of acetate amide-d3 in row 4 test tubes was aspirated to each of rows 6,7,8,9, and 10 test tubes. Next, an aliquot of 0.1ml of 10% v/v acetic anhydride in ethyl acetate and an aliquot of 0.1ml 1M sodium bicarbonate were added to rows 6,7,8,9, and 10 test tubes. The test tubes were gently shaked for 10 minutes and were aspirated into filters containing hydromatrix® powder that was sitting above test tubes of rows 11, 12, 13, 14, and 15. Aliquots of 1ml ethyl acetate were aspirated into filters and filtrates were collected in rows 11, 12, 13, 14, and 15 test tubes.

5. **Automated mass spectrometric analysis.**

Aliquots of 0.050ml of rows 4, 5, 11, 12, 13, 14, and 15 test tubes were placed in autosampler vials for mass spectrometric analysis. Total of 28 vials (1 to 28) were programmed for electrospray MS analysis using Q1 scan mode and product ion scan mode for reference standards and internal standards (vial 1 to 8), and using MRM mode for microsome reactions (vials 9 to 28). MRM mode of analysis or tandem MS analysis of 4 amines were set up as follows :

Amine 1 : acetamide $C_6H_5CH_2CH(CH_3)NH(COCH_3)$: 178.1 → 91.0;

acetamide-d3 $C_6H_5CH_2CH(CH_3)NH(COCD_3)$: 181.2 → 91.0;

Amine 2 : acetamide $C_6H_5CH_2CH(CH_3)NH(CH_3)(COCH_3)$: 192.2 → 91.0;

acetamide-d3 $C_6H_5CH_2CH(CH_3)NH(CH_3)(COCD_3)$: 195.2 → 91.0;

Amine 3 : acetamide $(OCH_2O)C_6H_3CH_2CH(CH_3)NH(CH_3)(COCH_3)$: 236.2 → 105.0;

acetamide-d3 $(OCH_2O)C_6H_3CH_2CH(CH_3)NH(CH_3)(COCD_3)$: 239.3 → 105.0;

Amine 2 : acetamide $(OCH_2O)C_6H_3CH_2CH(CH_3)NH(CH_2CH_4)(COCH_3)$: 250.0 → 105.0;

acetamide-d3 $(OCH_2O)C_6H_3CH_2CH(CH_3)NH(CH_2CH_4)(COCD_3)$: 253.2 → 105.0;

Ion ratios of acetamide to acetamide-d3 for amine 1 to 4 are as follows :

	0 min	30 min	60 min	90 min	120 min
Amine 1	0.45	0.31	0.33	0.28	0.27
Amine 2	0.35	0.22	0.21	0.23	0.19
Amine 3	0.36	0.20	0.22	0.22	0.21
Amine 4	0.92	0.60	0.69	0.80	0.72

6. **Calculation of percent of remained amine compounds**

Using the amount of amine at time t=0 as 100% , the % of remained amine with time is tabulated as follows :

	T= 0 min	30 min	60 min	90 min	120 min
Amine 1	100%	69%	73%	62%	60%
Amine 2	100%	63%	60%	65%	54%
Amine 3	100%	56%	61%	61%	58%
Amine 4	100%	65%	75%	87%	78%

[0020] This example demonstrates the one step simultaneous synthesis of deuterated acetamides by derivatization an authentic sample of a library of 4 chemical compounds having in common an amino group with labeled derivatizing reagent acetic anhydride-d6. These deuterated acetamides are used as labeled internal standards for the MS analysis of the remainder of the amines in stopped enzyme reactions as acetamides after being derivatized, also by a one step simultaneous chemical reaction, with acetic anhydride. The consequence of the one step reaction of these library compounds with acetic anhydride-d6 is the chemical attachment of a CD_3CO tagged group resulting in an increase in molecular mass of 45 amu, and the one step reaction with acetic anhydride tags on a CH_3CO group resulting in an increase in molecular mass of 42 amu. On the other hand, the same reaction with labeled acetic anhydride $(^{13}CH_3{}^{13}CO)_2O$ is anticipated to result in a mass increase of 44 amu, the size of the labeled tagged group $^{13}CH_3{}^{13}CO$. The difference between the use of the acetic anhydride reagent $(^{13}CH_3{}^{13}CO)_2O$ and the acetic anhydride-d6 $(CD_3CO)_2O$ in the above example is not just the difference in mass increase but the difference in the selection of enriched stable isotope atoms, carbon-13 (^{13}C) versus deuterium (D). Carbon-13 is sometimes preferred to deuterium in labeled internal standards because of its very low isotopic abundance compared to deuterium which has higher isotopic abundance. There are also other enriched stable isotope atoms commonly used in MS analysis besides carbon-13 and deuterium such as oxygen-18 (^{18}O) and nitrogen-15 (^{15}N), but the available labeled reagents containing these enriched isotope atoms are few. Therefore the selection of a labeled derivatizing reagent is based on the desire of mass increase and the desire of specific enriched stable isotope atom but is not based on the desired class of derivatized chemical compound such as amide, carbamate, etc. The presence of at least one common functional group out of the 4 functional groups mentioned above in a library of chemical compounds facilitates the selection of labeled derivatizing reagents described in this method. While the invention is particularly described with the illustrated example, it will be understood by those skilled in the art that the foregoing and other changes in details may be made therein without departing from the scope of the invention.